Note on the Comparative Effects on Tissues of Isotonic Saline and Distilled Water when used as Solvents for Mercuric Chloride and Formol in Histological Fixation.

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Introductory.

Should a simple fixative such as mercuric chloride or formol be dissolved in normal (i.e. isotonic) saline or in water? Curiously enough, observations are lacking on this simple but fundamental point.

Gustav Mann (**6**) states that formol should be diluted with isotonic saline 'because watery solutions cause such tissues as blood corpuscles and the central nervous system to swell up in whatever strength formol may be used'. That, moreover, is the only reference based (apparently) on actual observation which has come to my notice.

Most authorities on microscopical technique (Langeron, **3**; Mallory and Wright, **5**) advise that solutions of mercuric chloride and formol be prepared in distilled water, while Lee (**4**) advocates the dilution of formol with tap water, the salts in this tending to neutralize the free formic acid always formed after formol has been kept for any length of time. Finally, among practising histologists and pathologists there does not seem to be any consensus of opinion on the subject. Some consider that it is immaterial whether mercuric chloride or formol are dissolved in saline or water; others hold that it is 'safer' to make up these substances in saline.

The aim of these observations was to note the tissue-changes

(if any) caused by making up mercuric chloride or formol in distilled water or in isotonic saline.

Technique.

In these experiments every attempt was made to modify only the tonicity of the sodium chloride and that alone. To attain this end the following precautions were observed:

(1) The concentration of the substance employed as a fixing reagent was kept constant throughout the series, that of the mercuric chloride being 6 per cent., that of the formol 5 per cent. The solutions of the latter were all prepared from the same sample of commercial (40 per cent.) formol, since the strength of this substance is very liable to variation, especially after keeping. By 5 per cent. formol is meant a 5 per cent. solution of formaldehyde, i. e. a solution prepared by diluting one volume of 40 per cent. formol with seven volumes of water.

(2) The same volume of fixative (50 cc.) was always used.

(3) Care was taken in cutting out the pieces of tissue for fixation to keep them, so far as was possible, of the same size.

(4) The conditions of embedding and staining were kept constant. The tissues were dehydrated in ascending grades of alcohol—beginning with 50 per cent. alcohol—cleared, and embedded in paraffin all in exactly the same manner. To reduce cell-shrinkage during these processes to a minimum, the tissues were passed from absolute alcohol to a mixture of equal parts of absolute alcohol and xylol. They were then transferred to pure xylol, next to xylol-paraffin, and finally to pure paraffin. Further, all tissues were cut at the same thickness (8 μ), and, as a final precaution, the sections used for microscopic observation were taken after 50 cm. of the ribbon containing the sections had been cut on the microtome. In this way the sections were all taken from approximately the same depth beneath the surface of the piece of tissue—a point of some importance in view of the tendency of many fixatives to shrink the periphery more than the centre of tissues. Sections were stained on the slide with Ehrlich's haematoxylin and

eosin as a standard method, supplemented by Heidenhain's iron-haematoxylin followed by Lichtgrun.

(5) Finally, the question of personal bias (far more important than is generally supposed) was dealt with in the following manner: the identification numbers on the slides were covered with unmarked labels. The degree of tissue-change was then carefully noted for every slide in each series. Only then were the labels taken off the slides. I am convinced that methods such as these are necessary if minute differences in tissues either in response to variations in the fixative or, indeed, to any other factors, are to be accurately recorded.

It is usually impossible to check histological observations by quantitative methods. Only in certain specialized spheres, e.g. the counting of cells in body and other fluids, can this be done. It is therefore imperative in qualitative observations—such as those forming the subject of this note—to adopt every means whereby the conditions of experimentation can be standardized, and the personal factor reduced to a minimum.

The Comparative Effects of Using Normal Saline and Distilled Water as Solvents for Mercuric Chloride and Formol.

Amphibian and mammalian tissues were used for these observations. The following organs were studied:

In the Frog.—Liver and small intestine.

In the Cat.—Liver, duodenum, and kidney.

Liver was chosen because the relatively large size of the hepatic cells renders observation of their size and shape comparatively easy. The abundant blood in the sinusoids of this organ also enables the behaviour of the red blood-corpuscles to be noted.

Small Intestine was selected because it makes possible the study of two different tissues—epithelium and non-striated muscle—in the same section.

Kidney was studied partly because of the histological differences in the different portions of the urinary tubule, partly on account of the sensitivity of renal tissue to the action

of fixatives. For in this connexion it is a matter of common knowledge that fixed specimens of kidney frequently show swelling or (more usually) shrinkage of the renal epithelium.

The frogs were killed by pithing, the cats by a blow on the head. The tissues were fixed immediately after death.

Amphibian (Frog) Tissues.—The concentration of the normal (isotonic) saline was 0·6 per cent. Two frogs were used, i. e. the experiments were once repeated so as to observe whether the effects were constant.

(1) 6 per cent. Mercuric Chloride in 0·6 per cent. NaCl.—The preservation of both liver and intestine is normal. There is no evidence of either shrinkage or swelling of the cells. The shape of the red blood-corpuscles is normal.

(2) 6 per cent. Mercuric Chloride in Distilled Water.—Tissues fixed in this solution are indistinguishable from those fixed in no. 1.

(3) 5 per cent. Formol in 0·6 per cent. NaCl.—The fixation is normal and comparable to that obtained with mercuric chloride dissolved in the corresponding grade of saline (no. 1).

(4) 5 per cent. Formol in Distilled Water.—Liver.—The cells are normal in size and shape. Their cytoplasm is vacuolated, and the whole appearance suggestive of some change—probably of the nature of a partial solution of the cell-contents—brought about by fixation. There is no distortion of the red blood-corpuscles in the sinusoids. Intestine.—In some specimens the epithelium is normally preserved, in others the columnar epithelial cells are vacuolated and somewhat swollen. The muscular coats of the intestine are well preserved.

Mammalian (Cat) Tissues.—The concentration of the normal saline employed in this series was 0·9 per cent. The observations were once repeated, as for the frog series.

(1) 6 per cent. Mercuric Chloride in 0·9 per cent. NaCl.—Liver.—The fixation is normal. The red blood-corpuscles are not distorted. Duodenum.—Both intestinal epithelium and muscle are well fixed. Kidney.—The fixation

varies in the different segments of the renal tubules; the glomeruli are somewhat shrunken, while there is absence of shrinkage in the other portions of the renal tubules, i. e. the fixation is fair in the first and second convoluted tubules, in the ascending and descending portions of the loops of Henle, and in the collecting tubules.

(2) 6 per cent. Mercuric Chloride in Distilled Water.—Liver.—Fixation normal. There is no distortion of the red blood-corpuscles in the sinusoids. Duodenum.—Both epithelium and muscle are well preserved. Kidney.—Some glomerular shrinkage; the other elements of the renal tubules are normally fixed.

(3) 5 per cent. Formol in 0·9 per cent. NaCl.—Liver.—Cells normal in size and shape. No distortion of the red blood-corpuscles. Duodenum.—Epithelium well preserved; some shrinkage of the fibres in the muscle-layers. Kidney.—No distortion of the glomeruli; there are small areas in the medullary rays showing swelling of the tubule cells—especially in the loops of Henle. Sometimes the swelling of the renal epithelium is so marked that the lumina of the tubules are almost obliterated.

(4) 5 per cent. Formol in Distilled Water.—Liver.—There is neither shrinkage nor swelling of the cells. Examination of iron haematoxylin sections with the high power reveals faulty fixation of the ground cytoplasm, in that the latter has the appearance of having been partially dissolved by the fixative. The red blood-corpuscles in the sinusoids are not distorted. Duodenum.—Epithelium well preserved; slight shrinkage of the fibres in the muscle-layer. Kidney.—Both glomeruli and tubules are slightly shrunken.

Note on the Effect of using Hypertonic Saline as a Solvent for Mercuric Chloride and Formol.

To ascertain whether increasing the concentration of the NaCl would produce cell-shrinkage, observations were made on amphibian and mammalian tissues. The fixatives already

employed (and in the same proportion to the volume of the solvent) were made up in saline solutions of double the normal concentration, i. e. of 1·8 per cent. and 1·2 per cent. for the cat and frog respectively.

The results for both cat and frog tissues may be summarized as follows :

6 per cent. mercuric chloride and 5 per cent. formol dissolved in saline of double the normal concentration caused shrinkage of the tissues examined. The degree of shrinkage was notably greater in the formol than in the mercuric chloride series. Further, while intestinal epithelium is relatively tolerant to this increase in the tonicity of the NaCl, intestinal muscle and liver are less so. Kidney showed both glomerular and tubule shrinkage—especially in the formol series. The red blood-corpuscles of the cat were crenated and distorted, while those of the frog retained their normal shape. Finally, the mitochondria of the hepatic cells, after staining with iron haematoxylin, were found to remain unchanged no matter whether the fixative (mercuric chloride or formol) were made up in distilled water, isotonic saline, or hypertonic saline of double the normal concentration.

Conclusions.

It appears to be of no histological importance whether saturated (6 per cent.) solutions of mercuric chloride be dissolved in normal saline or in distilled water. No differences could be detected in specimens of liver, small intestine, and kidney fixed in either way, nor would there be any reason to expect such differences on a priori grounds. For the relatively high molecular concentration of the $HgCl_2$ is only very slightly altered by dissolving it in either isotonic saline or in hypertonic saline of double the normal concentration. In fact, the only effect of making up a concentrated solution of mercuric chloride in normal saline is slightly to increase the tonicity of the mixture.

In the case of a 5 per cent. solution of formol the evidence is that this reagent fixes tissues more faithfully when made

up in normal saline than in distilled water. When dissolved in the latter the ground cytoplasm is often vacuolated, and, sometimes, partly destroyed.

Subject to revision in the light of further observations, I suggest the following as an explanation of the distortion of tissues caused by fixation in 5 per cent. formol made up in distilled water. As already pointed out, the only effect of dissolving mercuric chloride in normal saline is *slightly to increase* the molecular concentration of the mixture. But in the case of formol this is different, for the low molecular concentration of 5 per cent. solution of formol (as compared to 6 per cent. $HgCl_2$) is *appreciably increased* by making it up in normal saline instead of distilled water. This means that while a given concentration of formol in normal saline may be isotonic with tissues, the same concentration of formol in distilled water may be sufficiently hypotonic to cause distortion and swelling of cells.

Solutions of mercuric chloride and formol when dissolved in hypertonic solutions of saline of double the normal strength give rise to tissue-shrinkage. This shrinkage is more marked in the formol than in the mercuric chloride series. Further, the degree of shrinkage induced by the fixative varies greatly—as is well known—with different tissues.

These remarks concerning the dilution of mercuric chloride and formol in distilled water and in isotonic saline only apply to these two fixing reagents used in the concentrations already mentioned, these being, moreover, the concentrations at which they are the most commonly employed. The question of the tonicity of compound fixatives is omitted, for here, as claimed by Gatenby (**1** and **2**), the strength of the fixative is regulated by diluting it, if necessary, with distilled water. Thus, if fixation in a chrome-osmium mixture produces cell-shrinkage, the fixative should be diluted with a known volume of distilled water. This trial and error method is repeated until the dilution of the fixative is such that it does not cause shrinkage by a too rapid exosmosis from the cells.

The practical outcome of this note, then, is that while it is immaterial whether a concentrated (6 per cent.) solution of mercuric chloride be dissolved in isotonic saline or in distilled water, formol of 5 per cent. should be made up in isotonic saline and NOT in distilled water.

In conclusion, I have to thank Professor Sir Charles Sherrington for his interest in my work and for according me every facility in his laboratory.

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